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Award Number: DAMD17-97-1-7122

TITLE: Cell Type-Specific mRNA Amplification and Expression
Profiling from Breast Tumor Sections

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REPORT DATE: October 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
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20010723 089

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE
October 2000

3. REPORT TYPE AND DATES COVERED
Annual (30-Sep-99 - 29-Sep-00)

4. TITLE AND SUBTITLE

Cell Type-Specific mRNA Amplification and Expression Profiling from Breast Tumor Sections

5. FUNDING NUMBERS
DAMD17-97-1-7122

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION
REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING
AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The evolution of solid tumors involves acquisition of genetic abnormalities, which result in changes in both the set of genes expressed and the relative levels of gene expression. However, the increasing number of candidate genes whose expression needs to be evaluated for prognostic, diagnostic, therapeutic, or research purposes will require obtaining material from numerous tissue sections. Therefore this proposal is motivated by the need for more effective use of clinical specimens, and will address the problem of obtaining sufficient and cell type specific mRNA from clinical breast tumor specimens. This will entail adapting/developing procedures to amplify with fidelity the mRNA repertoire expressed in small numbers of normal, pre-cancerous and malignant breast epithelia. To this end, in this project period, we have concentrated effort on establishing and validating microarray-based assays for measuring gene expression levels and have demonstrated the capability to isolate and amplify mRNA from cultured cells. Realization of these objectives will allow, in the future, development of a resource, consisting of amplified mRNA populations from individual cells from normal and tumor material, that can be used for evaluation of the prognostic, diagnostic and/or therapeutic importance of genes expressed in breast cancer.

14. SUBJECT TERMS

Breast Cancer

15. NUMBER OF PAGES

13

16. PRICE CODE

17. SECURITY CLASSIFICATION
OF REPORT

Unclassified

18. SECURITY CLASSIFICATION
OF THIS PAGE

Unclassified

19. SECURITY CLASSIFICATION
OF ABSTRACT

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

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Donna J. Alderson 4/3/01
PI - Signature Date

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INTRODUCTION:

The evolution of solid tumors involves acquisition of genetic abnormalities, which result in changes in both the set of genes expressed and the relative levels of gene expression. Therefore it is desirable to be able to both characterize and compare the levels of expression of particular genes in normal and tumor cells. Currently, assays of gene expression are carried out on mRNA isolated in bulk from tissue specimens, or at the individual cell level by *in situ* hybridization or immunohistochemistry. However, neither approach will meet all the needs of the research and clinical communities. Conventional mass biochemical extraction procedures are not appropriate in breast cancer, because extraction of mRNA from single cell types is difficult due to the intermingling of epithelial and stromal components, and the fact that the amount of malignant or pre-malignant tissue available in the specimen is small. In addition, very few cells in the specimen make up the normal ductal epithelium. Cell type-specific gene expression can be visualized in tissue sections, but there are now increasing numbers of candidate genes to be evaluated for prognostic, diagnostic, therapeutic, or research purposes, and expression analysis of all these genes will require numerous individual tissue sections. Therefore this proposal is motivated by the need for more effective use of clinical specimens, and will address the problem of obtaining sufficient and cell type specific mRNA from clinical breast tumor specimens for analysis of gene expression in normal and diseased tissue. This will entail adapting/developing a new approach to archiving the repertoire of genes expressed in normal, pre-cancerous and malignant breast epithelia. Procedures used to obtain gene expression profiles from single neurons (Eberwine *et al.*, 1992) will be adapted for use with clinical breast cancer specimens, allowing amplification of the mRNA repertoire from small numbers of cells from normal ductal epithelium, DCIS and invasive carcinoma. The procedure has been reported to provide 10^6 fold amplification and uses an oligo-dT primer incorporating the promoter binding site for T7 polymerase to prime first strand cDNA synthesis. Subsequently linear amplification of this small quantity of cDNA is accomplished by *in vitro* transcription using T7 RNA polymerase (Figure 1). Thus, this procedure could provide enough material for multiple and diverse assays of gene expression and/or for the generation of cDNA libraries. Therefore the purpose of this proposal is to develop the capability to isolate and amplify with fidelity total mRNA from small numbers of microdissected cells of histologically defined types. We will then apply these procedures to obtain expression profiles for cells representing normal epithelium, DCIS and invasive carcinoma from frozen and paraffin embedded sections of tumors. Our objectives are to: (1) Demonstrate linear amplification of high complexity RNA from a homogeneous population of cells; optimize the techniques to maximize the amount and complexity of amplification that can be obtained while preserving relative copy number of different mRNA species, and (2) apply these techniques to amplify mRNA from microdissected cells from frozen and formalin fixed sections containing normal ductal epithelial cells, DCIS and invasive carcinoma, and use this material to obtain expression profiles for these different cell types. Realization of these objectives will allow development of a resource, consisting of amplified mRNA populations from individual normal and tumor-specific material, that can be used for evaluation of the prognostic, diagnostic and/or therapeutic importance of genes expressed in breast cancer.

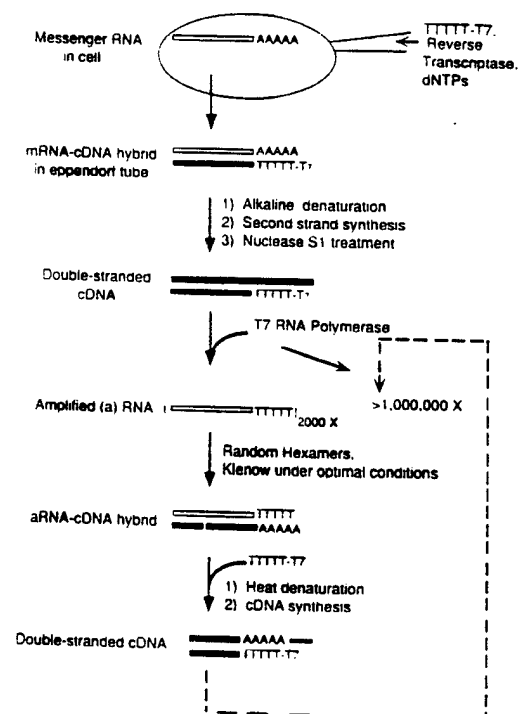


Figure 1. aRNA amplification scheme (from Eberwine *et al.*, 1992)

BODY:

A. Reporting Period.

The work described herein was performed over the period from 9/29/99 to 9/29/00.

B. Progress

Work in this period has focused on Technical Objective 7. Linear amplification methods allow detection of gene expression from smaller amounts of starting material. However, they must be assessed for biases introduced during the amplification and labeling processes.

Statement of Work

1. Technical Objective 1. Demonstrate linear amplification of high complexity aRNA (amplified antisense RNA) from a homogeneous population of cells, the breast tumor cell line BT474.

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| Task 1 | Months 1-3 | Validate assay for expression levels. Make test RNA population by transcribing test genes <i>in vitro</i> , label and hybridize to array of test clones. Demonstrate linearity of the assay. |
| Task 2 | Months 4-9 | Grow BT474 cell cultures, isolate mRNA, measure expression levels of test genes in mRNA isolated from BT474 and estimate complexity by hybridization to IMAGE cDNA array. |
| Task 3 | Month 4-9 | Carry out amplification on various amounts of bulk BT474 mRNA down to 0.1 pg, measure expression levels of test genes in aRNA and hybridize to IMAGE cDNA array. |
| Task 4 | Months 10-13 | Prepare frozen and fixed samples of BT474 cells and cut sections. Carry out mRNA amplification on sections of BT474 cells, measure expression levels of test genes in aRNA and estimate complexity by hybridization to IMAGE cDNA array. |
| Task 5 | Months 10-13 | Prepare fluorescently labeled probes for test genes and carry out <i>in situ</i> hybridization to sections of BT474 cells. Measure intensity of fluorescent hybridization signals and determine relative levels of expression of test genes in the cells in the sections. |
| Task 6 | Months 10-13 | Compare relative levels of expression of test genes in aRNA and <i>in situ</i> as determined by FISH in Task 7. Compare results of hybridization to IMAGE cDNA array with aRNA made to whole sections and microdissected cells. |
| Task 7 | Months 10-13 | Optimize protocols (Tasks 4-6). |

2. Technical Objective 2. Apply the techniques from Objective 1 to amplify mRNA from microdissected cells from frozen and formalin fixed sections containing normal ductal epithelial cells, DCIS and invasive carcinoma. Use this material to obtain expression profiles for these different cell types using SAGE and hybridization to an array of clones from the IMAGE cDNA library.

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| Task 8 | Months 14-24 | Prepare fluorescently labeled probes for test genes and carry out <i>in situ</i> hybridization to breast tumor sections. Measure intensity of fluorescent hybridization signals and determine relative levels of expression of test genes in different cell types in the tumor section. |
| Task 9 | Months 14-24 | Carry out mRNA amplification on tumor sections. |

Task 10	Months 14-24	Measure expression levels of test genes in aRNA from tumors.
Task 11	Months 14-24	Compare relative levels of expression of test genes in aRNA and <i>in situ</i> as determined by FISH in Task 8.
Task 12	Months 14-24	Carry out expression analysis on aRNA from tissue sections by hybridizing the aRNA to an array from the IMAGE cDNA library.

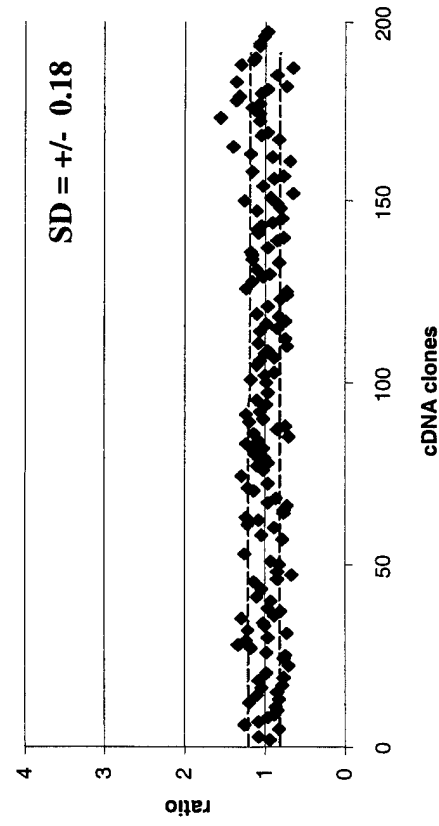
Task 7. Optimize protocols.

Linear amplification methods allow detection of gene expression from smaller amounts of starting material. However, they must be assessed for biases introduced during the process. We have investigated two sources of bias, including those introduced during the amplification procedure itself and during the labeling reaction. We are using cDNA microarrays to measure and evaluate the linearity and uniformity of the aRNA amplification procedure. In this assay, differentially fluorescently labeled probes were made from two nucleic acid populations. One mRNA is labelled with one fluorochrome (e.g. Cy3, red) and a second RNA with another fluorochrome (e.g. fluorescein, green). These probes are then hybridized to the microarray and the fluorescence intensity of each probe is determined on each array spot. The red:green ratios indicate the similarities and differences in the level of expression of a particular gene in the two RNA populations.

- a. Comparison of two different labeling methods. We evaluated direct labeling in which Cy3 or Cy5 labeled nucleotides are incorporated into the probe during the reverse transcriptase (RT) reaction and the use of amino-allyl labeling, an indirect method. For amino-allyl labeling the amino-allyl modified nucleotide is incorporated into both the test and reference by the RT reaction and subsequently the two populations are differentially labeled with Cy3 and Cy5 by a chemical reaction. The potential advantage of the indirect method is that the RT enzyme is incorporating the same nucleotide into the test and reference populations, and therefore may avoid biases due to different efficiencies of incorporation into specific sequences. To compare the two labeling method, an RNA population was divided. One half was labeled with Cy3 dCTP and the other with Cy5 dCTP. A second population was labeled by the amino-allyl procedure. Two self vs. self hybridizations were carried out to a microarray. If the RNA had been labeled equally with Cy3 or Cy5, then all genes in the array should be represented at equal levels in the two RNA populations, and the red:green intensity ratios should be constant across all spots. Deviant ratios indicate non-uniform labeling of those particular genes. Figure 2. shows the results of these comparisons. The amino-allyl labeling procedure resulted in a smaller distribution in the self vs. self ratios, indicating more that fewer biases in labeling are introduced by this procedure compared to the direct labeling.
- b. Reproducibility of amplification procedure. Three independent amplifications of an RNA population were carried out and then compared to each other by microarray analysis. Although the results show high correlations (Figure 3), there is a greater variation in the ratios than in the self vs. self comparison.
- c. Comparison of unamplified and amplified RNA populations. RNA from two breast cancer cell lines was amplified. The amplified and unamplified starting materials were labeled and hybridized to microarrays. High level expression of ERBB2 in BT474 compared to MCF7 was observed in both the unamplified and amplified samples, as

expected. However, the normalized ratio of ERBB2 expression in the unamplified sample was greater than in the amplified sample (Figure 4, ratio = 9 compared to 7), indicating that the procedure may not quantitatively preserve relative gene expression.

Direct incorporation



Amino-allyl labeling

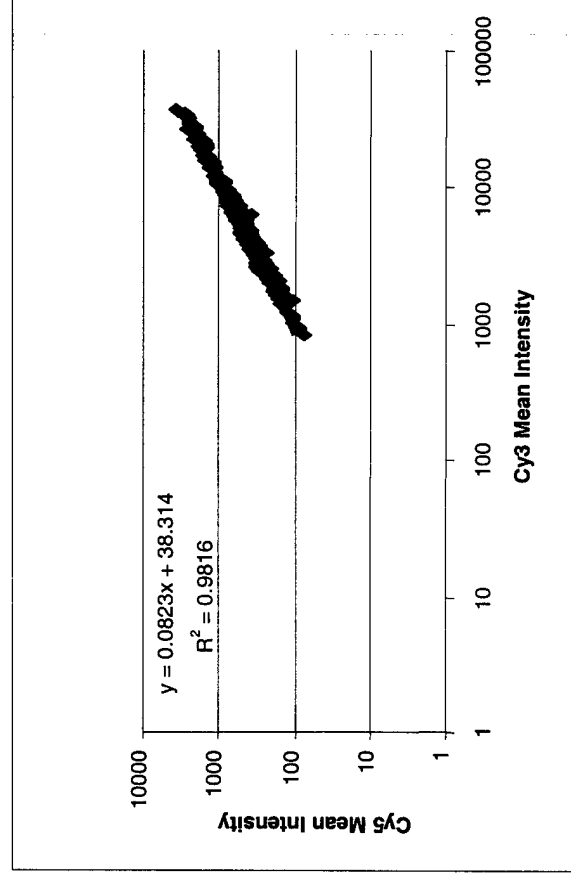
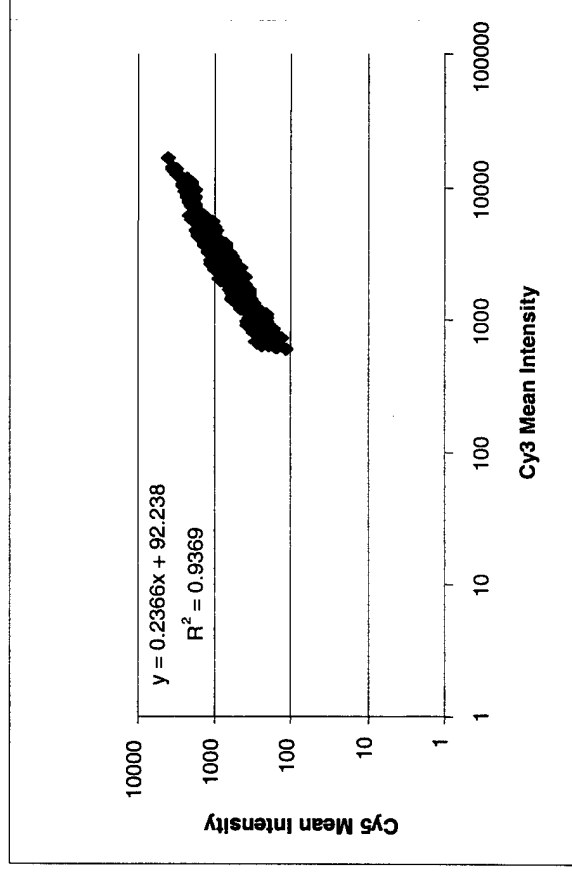
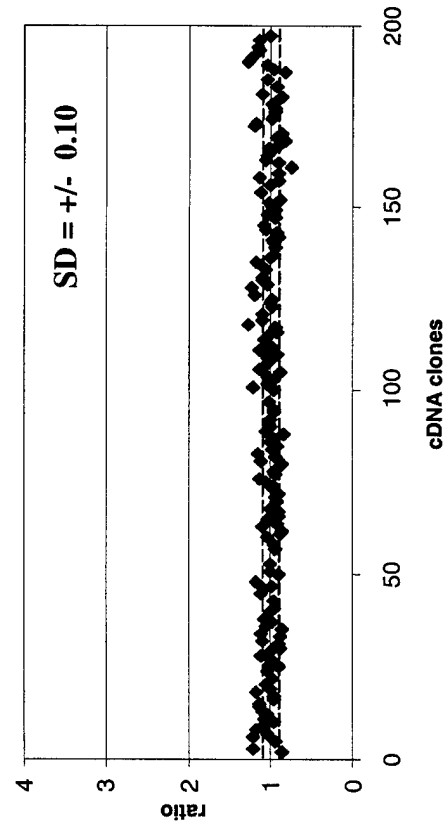


Figure 2. Self vs. self comparison of RNAs labeled by two different methods. See text for details.

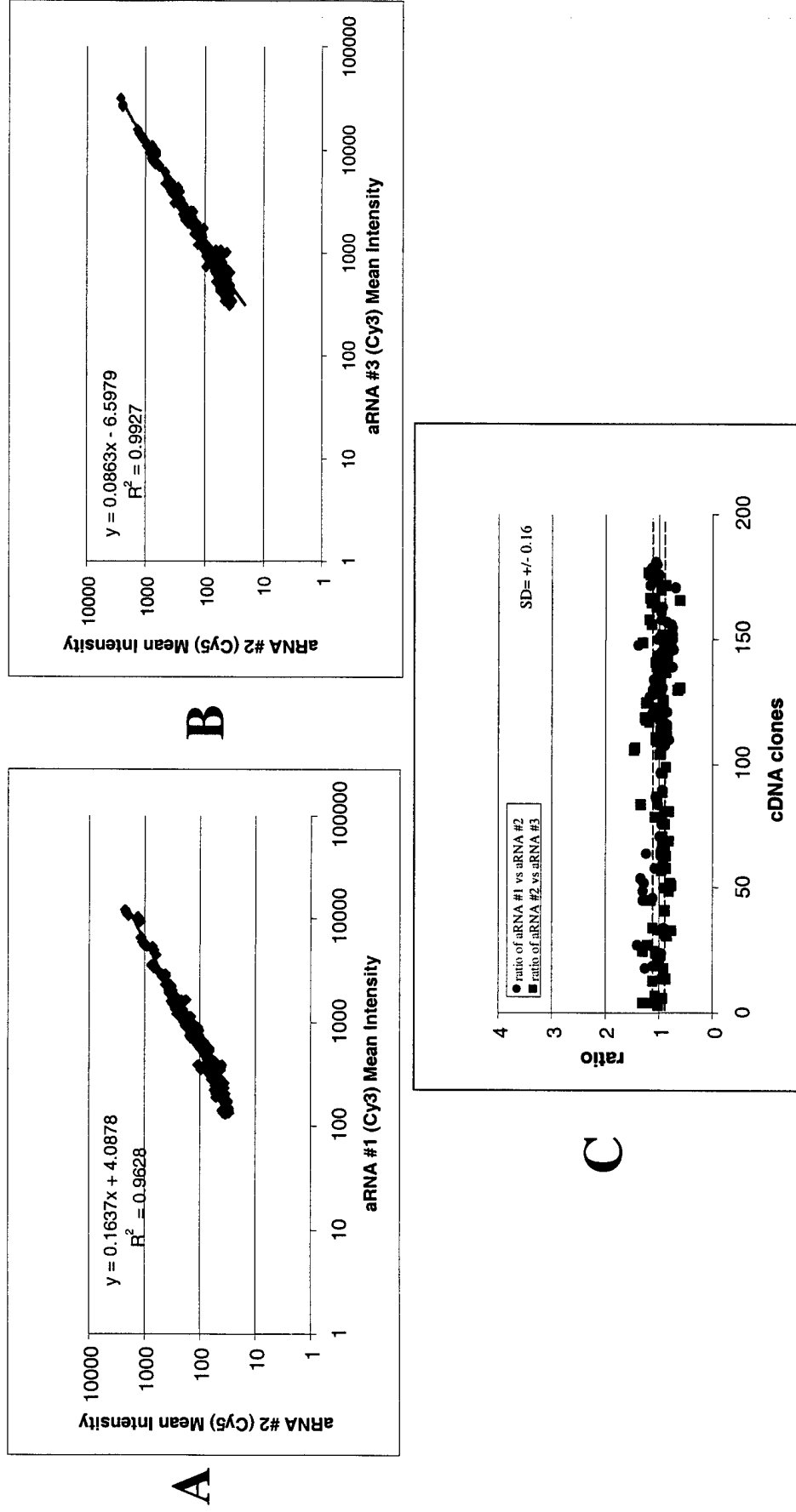


Figure 3. Self vs Self: using 3 independent RNA amplifications Correlation between the hybridization results obtained for 3 independent amplification prepared from total RNA: **A**) 250 ng aRNA #1(Cy3) vs 250 ng aRNA #2 (Cy5) **B**) 250 ng aRNA #3 (Cy3) vs 250 ng aRNA #2 (Cy5) and **C**) combined ratios for aRNA #1 vs aRNA #2 and for aRNA #3 vs aRNA #2

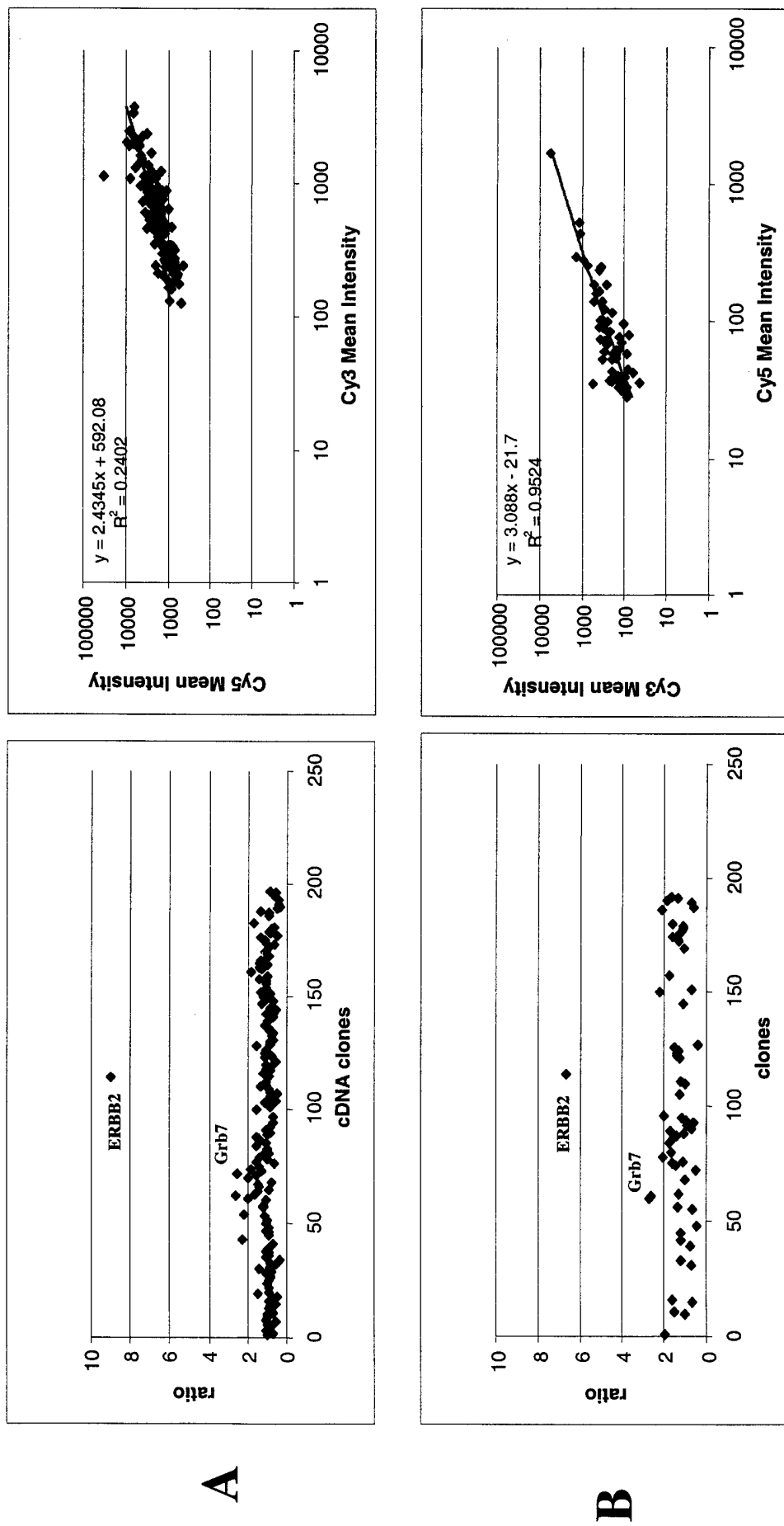


Figure 4. MCF7 vs BT474: comparison of unamplified and amplified RNA. Hybridization results obtained from **A**) 10 µg BT474 Total RNA (Cy3) vs 250 ng aRNA from BT474 (Cy3) and **B**) 250 ng aRNA from MCF7 (Cy5) vs 10 µg MCF7 Total RNA (Cy5)

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstration of 10% standard deviation in self vs. self ratios on microarrays using amino-allyl labeling.
- Independent amplification reactions of a single RNA population show high correlations, but greater variations in microarray ratios than unamplified self vs. self comparisons.
- Demonstration of preservation of relative gene expression levels after amplification. However, expression levels are not quantitatively preserved.

REPORTABLE OUTCOMES:

None

CONCLUSIONS:

Work in this project period was focused on investigating labeling and amplification by the Eberwine procedure (Eberwine et al, 1992). cDNA microarrays were used to evaluate the linearity and fidelity of the aRNA amplification procedure and to begin optimization of the aRNA amplification. The results described above indicate that amino allyl labeling introduces fewer biases into the labeling procedure, and therefore it is the preferred method for labeling for microarray analyses. The work described above has also demonstrated that the amplification procedure introduces variation into the measurements. The procedure has not been demonstrated to result in quantitative preservation of ratios. For some applications, the procedure will be adequate if a qualitative assessment of relative gene expression is sufficient. However, one should use caution when interpreting microarray data obtained after amplification.

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Eberwine, J., Yeh, H., Miyashiro, K., Cao, Y., Nair, S., Finnell, R., Zettel, M. and Coleman, P. 1992. Analysis of gene expression in single live neurons. Proc. Natl. Acad. Sci. U. S. A. 89, 3010-3114.

APPENDIX:

None